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Hypoxia enhances tenocyte differentiation of adipose-derived mesenchymal stem cells by inducing hypoxia-inducible factor-1α in a co-culture system

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Abstract

Objectives: Tissue engineering is a promising approach for repair of tendon injuries. Adipose-derived mesenchymal stem cells (ADMSCs) have gained increasing research interest for their potential in improving healing and regeneration of injured tendons. The present study aimed to investigate effects of O_2 tension and potential signalling pathways on AMDSC differentiation into tenocytes, in an indirect co-culture system.

Materials and methods: Human ADMSCs were co-cultured under normoxia (20% O_2) and also under hypoxia (2% O_2). Tenocyte differentiation of AMDSCs and expression of hypoxia-inducible factor-1 (HIF-1α) were analysed by reverse transcription-PCR, Western blotting and immunohisto chemistry. Furthermore, HIF-1α inhibitor and inducer (FG-4592) effects on differentiation of AMDSCs were studied using qPCR, immunofluorescence and Western blotting.

Results: Indirect co-culture with tenocytes increased differentiation of ADMSCs into tenocytes; furthermore, hypoxia further enhanced tenocyte differentiation of AMDSCs, accompanied by increased expression of HIF-1α. HIF-1α inhibitor attenuated effects of hypoxia on differentiation of ADMSCs; in contrast, FG-4592 increased differentiation of ADMSCs under both hypoxia and normoxia.

Conclusions: Taken together, we found that growing ADMSCs under hypoxia, or activating expression of HIF-1α to be important in differentiation of ADMSCs, which provides a foundation for application of ADMSCs in vivo for tendon regeneration.

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Introduction

Tendon injuries of the hand or foot are associated with considerable socioeconomic costs and patient disability. Many attempts have been made to treat tendon injuries both in humans and horses, but the results have been largely unsatisfactory with a high risk of re-injury (1). This condition reflects the poor healing properties of tendon tissue due to its low vascularization and scarce cell content and requires the application of various tendon grafting procedures. However, the supply of autologous grafts is limited, and graft harvesting is associated with donor-site morbidity (2,3). Therefore, tissue engineers seek to address this through implantation of cell-seeded scaffolds to fill these defects and accelerate formation of new tendon tissue.

Bone marrow-derived mesenchymal stromal cells (BMSCs) are the most commonly used cells in tissue engineering, and the implantation of BMSCs into tendon injury has provided promising results in several in vivo studies; however, the low yield of stem cells may be a limitation in both pre-clinical and clinical applications. Adipose-derived stem cells (ADMSCs), which were first identified in 1980s, have quickly excelled in the cell-based tissue regenerative field because of their abundant volume and the ease with which they are obtained. Moreover, ADMSCs have demonstrated their ability to differentiate towards the adipogenic, myogenic (4), osteogenic (5,6) and chondrogenic lineages (7). In addition, it has been reported that ADMSCs have numerous advantages over BMSCs because they are readily available, easy to extract and isolate and do not produce

post-operative complications (8). Therefore, ADMSCs appear to be the most promising stem cell population to have clinical relevance as an alternative for tendon repair.

Co-culture of MSCs with various cell types has promoted proliferation and induced their differentiation into distinctive lineages (e.g. neurogenic, myogenic, osteoblasts or chondrogenic) (6,9,10). In the last few years, co-culture systems have been studied to simulate the in vivo microenvironment and to induce stem cell differentiation, which is probably caused by the paracrine effects of factors released directly by cells (11). Although the expansion and regenerative potential of ADMSCs are influenced by multiple factors such as serum contents, basal medium type, glucose concentration, stable glutamine, cell-plating density, and plastic surface quality (11–14), hypoxia appears to play a key stimulating role during ADMSCs proliferation and differentiation (15–17). Hypoxia alters cellular functions by regulating the "master" transcription factor HIF-1α, and the "master" microRNA miR-210. ROS generation is heavily involved in controlling the cellular and molecular alteration of ADMSCs during hypoxia. HIF-1α is one of the subunits of heterodimeric transcription factor, HIF-1. Under a low oxygen level, HIF-1α is stabilized and translocates into the nucleus, where it dimerizes with HIF-1β, another subunit of HIF-1, to form the HIF-1 complex, which then binds to the hypoxia-responsive elements (HREs) in the promoters of target genes (18,19). Although HIF-1α is not involved in the proliferation or migration of ADMSCs under hypoxic conditions, it is a master regulator of a plethora of genes involved in differentiation and angiogenesis. However, the cellular and molecular mechanisms involved in the stimulation of ADMSCs during hypoxia are not fully understood.

In the present study, we have aimed to investigate whether ADMSCs could be induced into tenocytes-like cells by indirect co-culture with tenocytes in vitro. In addition, we investigated if the low oxygen tension may improve the differentiation of ADMSCs and the involvement of HIF-1α in differentiation of ADMSCs towards tenocytes in hypoxic conditions by using HIF-1α inhibitor and inducer respectively.

Materials and methods

ADMSCs culture

Human ADMSCs were purchased from Cyagen biotechnology company (Guangzhou, China) and cell culture was performed according to the supplier's specifications. Cells were cultured in the Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% antibiotic mixture (penicillin and streptomycin) (Solarbio, Shanghai, China) at 37 °C in a humidified 5% CO_2 incubator. The culture medium was discarded and replaced with fresh medium every 3–4 days. Cells of three to six passages were used for the subsequent experiments.

Harvest and Culture of Tenocytes

Adult female Sprague–Dawley rats (220–250 g) purchased from Animal Center of Chinese Academy of Sciences, Shanghai, China, were used for tenocytes harvest. The protocol for animal care and use conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the Animal Care and Use Committee of Wenzhou Medical University. Primary tenocytes were obtained by slaughtered rats. The samples of the Achilles tendon were separated from rats' hindlimb and cut into pieces with the size of around 1 mm^3 while carefully removing the epi- and peritendon sheath. The tissue pieces were immersed in 10 ml of 0.2% collagenase II solution at 37 °C for 5 h under gentle agitation. The digested tissue was then gently filtered through a sterile 200 μm filter using a sterile 3 cm^3 pipette. The resulting filtrate was centrifuged at 1000 rpm for 5 min and the supernatant was descanted. The cell pellet was resuspended in 1 × PBS and re-centrifuged at 1000 rpm for 5 min. Following this wash, the cells were resuspended in DMEM/F12 (Gibco) with 15% FBS (Gibco) and 1% antibiotic mixture (Penicillin and Streptomycin) (Solarbio). The cells and media was then plated and incubated at 37 °C and 5% CO_2. Cells were washed and fed every 3 days and passaged when 80–100% confluent. Tenocytes between passages 3 and 5 were used for immunofluorescence analysis and for co-cultural experiments.

Indirect co-culture (standard culture condition)

The co-culture system was constructed with a transwell chamber (pore size 0.4 μm; Coast, NY, USA) which can be inserted into the well of six-well or one-well plates. ADMSCs were seeded on polystyrene plates at 1×10^4/well, while tenocytes were seeded on the membrane (with pore size of 4 μm) of the transwell chamber at the ratio of 1:1. The chambers were inserted into the well of the plate allowing the crosstalk of the two cell types. Incubation was performed in DMEM/F-12 medium containing 15% FBS in 5% CO_2. Half of the culture medium was changed every 3 days. All the experiments were performed in triplicate and evaluations were performed 0, 7, 14 and 21 days of cultures.

HIF-1 inhibition and inducer

To inhibit the activity of HIF-1α, a HIF-1α inhibitor (6,20) was used. The inhibitor of HIF-1α 3-[2-(4-adamantan-1-yl-phenoxy)-acetylamino]-4-hydroxybenzoic acid methyl ester was obtained from Calbiochem (Bad Soden, Germany). To stabilize the activity of HIF-1α, a HIF-1α stabilizer Roxadustat (FG-4592) one of [(4-Hydroxyl-benzo[4,5]thieno[3,2-c]pyridine-3-carbonyl)-amino]-acetic acid derivatives was used. This class of compounds showed the ability to stabilize HIF-1α by inhibition of prolyl hydroxylase (PHDs) (21). Roxadustat (FG-4592) was obtained from Selleck Chemicals LLC. Cells were treated with HIF-1α inhibitor at a concentration of 60 μM and HIF stabilizer Roxadustat (FG-4592) at a concentration of 5 μM in both 20% and 2% O_2 samples.

Exposure to sustained hypoxia in vitro

Hypoxic conditions were attained using an anaerobic chamber (Thermo 1029, California, USA). The oxygen level was maintained at 2% using a gas mixture of 2% oxygen, 93% N_2 and 5% CO_2, as previously described (Choi *et al.* 2014). In subsequent experiments, HIF-1 inhibition and inducer were each randomly allocated into normoxic conditions (20% O_2) or hypoxic conditions. Hence, six groups were evaluated and compared: (i) normoxic conditions and hypoxic conditions; (ii) HIF-1 inhibitor under normoxic conditions and hypoxic conditions; (iii) FG-4592 under normoxic conditions and hypoxic conditions.

Real-time polymerase chain reaction

Quantitative real-time PCR (qPCR) analyses were performed on co-cultured ADMSCs after 0, 7, 14 and 21 days of incubation in order to compare the expression of specific genes, outlined in Table 1. Total mRNA was extracted from the native ADMSCs and differentiated ADMSCs by using TRI Reagent (Invitrogen) according to the manufacturer's instructions. cDNA were synthesized from total RNA using a random primer and the RevertAid First Strand cDNA Synthesis Kit (Thermo, Lithuania). qPCR was then performed to measure and compare relative expression levels of collagen type I (COL1), collagen type III (COL3), tenomodulin (TNMD), thrombospondin-4 (THBS4), Scleraxis (SCX), HIF-1α and glycosaminoglycan (GAG) using the CFX96TM Real-Time PCR system equipped with CFX Manager 3.0 software (Bio-Rad, Hercules, CA, USA). The reaction mixtures were incubated for 3 min at 95 °C, followed by 95 °C for 10 s, 60 °C for 30 s, 95 °C for 45 s and 72 °C for 7 min. For each gene, a reaction mixture with water instead of cDNA template was run at the same time as a PCR negative control. The relative expression levels were normalized by the transcriptional levels of 18S rRNA, and analysed with Bio-Rad CFX Manager 3.0 Analysis software. Each PCR reaction was performed in triplicate independently.

Western blot analysis

Briefly, cells were homogenized in ice-cold lysis buffer containing 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 10 mM

Table 1. Primers Used in RT-PCR Reactions

Gene	Primer sequences	Accession no.	Product size (bp)
COL I	F:GAGAGTACTGGATCGACCCTAACC R: CTGACCTGTCTCCATCTTGCA	NM_053304.1	77
COL III	F:GTGTCTGCGACTCGGGATCT R:TAGAAGGCTGTGGACATATTGCA	NM_032085.1	120
TNMD	F:GGACTTTGAGGAGGATGGTGAA R:GGACCACCCATTGCTCATTC	NM_022290.1	83
THBS 4	F:CAACTGCCCCACCGTCAT R:TGGGATGCCGTCATTATCGT	NM_017133.1	97
SCX	F:ATCCCGACCGAGCCAGCAGA R:ACCGAGGCTTCACCCACCA	NM_001130508.1	111
HIF-1a	F: TGGACTCTGATCATCTGACC R: CTCAAGTTGCTGGTCATCAG	NR_046237.1	601
GAG	F: TAGAGAAGAAGAGGGGTTAGG R: AGCAGTAGGAGCCAGGGTTAT	NM_022190.1	322
GAPDH	F: GAGCCACATCGCTCAGACAC R: CATGTAGTTGAGGTCAATGAAGG	NM_012924.2	450

F, Forward; R, Reverse.

Na2P2O7, 10 mM NaF, 1 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM sodium vanadate and 1 mM PMSF. Cell homogenates were incubated for 15 min at 4 °C, and centrifuged at 12,000 rpm, for 15 min at 4 °C. The equivalent of 50 μg of total protein was loaded onto SDS-PAGE and transferred to PVDF membrane (Bio-Rad). The membrane was blocked with 5% non-fat milk in TBS with 0.1% Tween 20 for 90 min, and then incubated overnight at 4 °C with primary antibody (COL1 and HIF-1α 1:300; Santa Cruz Biotechnology, California, USA; COL3 and TNMD 1:800; Cell Signaling Technologies, Danvers, MA, USA; THBS4 and SCX 1:1000; Abcam, Cambridge, UK) solutions according to the manufacturer's recommendations. Then the membranes were washed with TBS for 5 min three times and primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies. Signals were visualized using the ChemiDicTM XRS + Imaging System (Bio-Rad). Experiments were repeated three times.

Immunofluorescence

Immunofluorescence staining was performed on co-cultured ADMSCs for 21 days. For immunostaining, cells contained in each well were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for 15 min at room temperature. Then, the cells were blocked with 5% rabbit serum albumin or 5% goat serum albumin in PBS for 1 h at RT, then incubated overnight at 4 °C with either goat polyclonal anti-COL I (1:200; Santa Cruz Biotechnology) or rabbit polyclonal anti-HIF-1α (1:200; Santa Cruz Biotechnology) in PBS. Following another PBS wash, each well was incubated with Alexa Fluor 488 rabbit-anti-goat secondary antibody or Alexa Fluor 594 goat-anti-rabbit secondary antibody (1:200; Molecular Probes, Eugene, OR, USA) in PBST (Phosphate Buffer Solution+Twen-20) for 2 h at RT. The nuclear dye 4′,6-diamidino-2-phenylindole (DAPI) (KeyGEN, Nanjing, China) was used for identifying the cell nuclei. Each well of the plate was observed with an inverted confocal microscope (Nikon, Tokyo, Japan).

Statistical analysis

Data are presented as the mean ± standard deviation from three independent experiments. Statistical significance was examined using Student's *t*-test when there were two experimental groups. When more than two groups were compared, statistical evaluation of the data was performed using one-way analysis of variance (ANOVA) and Dunnett's *post hoc* test. $P < 0.05$ were considered statistically significant.

Results

Hypoxia increases differentiation of ADMSCs to tenocytes in indirect co-culture

To evaluate the effect of hypoxia on the differentiation ability of the ADMSCs to tenocytes in indirect co-culture, the gene expression levels of COL I, COL III, TNMD, THBS 4, SCX and GAG were determined using qPCR in normoxia and hypoxia at 7, 14 and 21 days post-induction. As shown in Fig. 1, hypoxia treatment significantly increased the gene expression levels of COL I, COL III, TNMD, THBS 4, SCX and GAG respectively within 7–21 days, 14–21 days, 21 days, 14–21 days, 14–21 days, 21 days after co-culture with tenocytes, compared to normoxia-treated controls ($*P < 0.05$, $**P < 0.01$). On day 21, the expression level of genes COL I was at 45.01 ± 2.07 in hypoxia versus 27.88 ± 0.09 in normoxia, COL III expression was at 60.67 ± 2.12 in hypoxia versus 36.83 ± 1.92 in normoxia, GAG expression was at 46.37 ± 1.12 in hypoxia versus 32.06 ± 1.45 in normoxia, SCX expression was at 50.43 ± 1.61 in hypoxia versus 27.26 ± 1.82 in normoxia, THBS 4 expression was at 52.46 ± 1.73 in hypoxia versus 26.17 ± 2.02 in normoxia and TNMD expression was at 51.67 ± 1.64 in hypoxia versus 37.75 ± 1.27 in normoxia (Fig. 1a–f). After 21 days of transwell co-culture with tendon pieces, the ADMSCs in hypoxia became more elongated, slender with thinner processes, with a tenocyte-like shape and distribution (Fig. 1g). These results imply that hypoxia can effectively increase the differentiation of ADMSCs to tenocytes in indirect co-culture.

Indirect co-culture with tenocytes increases the differentiation of ADMSCs to tenocytes and hypoxia treatment further strengthens the differentiation

Co-culture plays an essential role in the differentiation of ADMSCs into specific cell types. After 21 days of transwell co-culture with tendon pieces, the treated cells became more elongated, slender with thinner processes, with a tenocyte-like shape and distribution (data not shown). Next, we examined the alterations of tenocytes-associated proteins markers including COL I, COL III, TNMD, THBS 4 and SCX in both ADMSCs alone and co-culture with tenocytes and the effect of hypoxia on these alterations by Western blot. Western blot results showed that (Fig. 2a–d) the levels of tenocytes-associated proteins of markers (COL I, COL III, TNMD, THBS 4 and SCX) were significantly increased in indirect co-culture cells in both normoxia and hypoxia conditions. Furthermore, hypoxia-treated groups showed

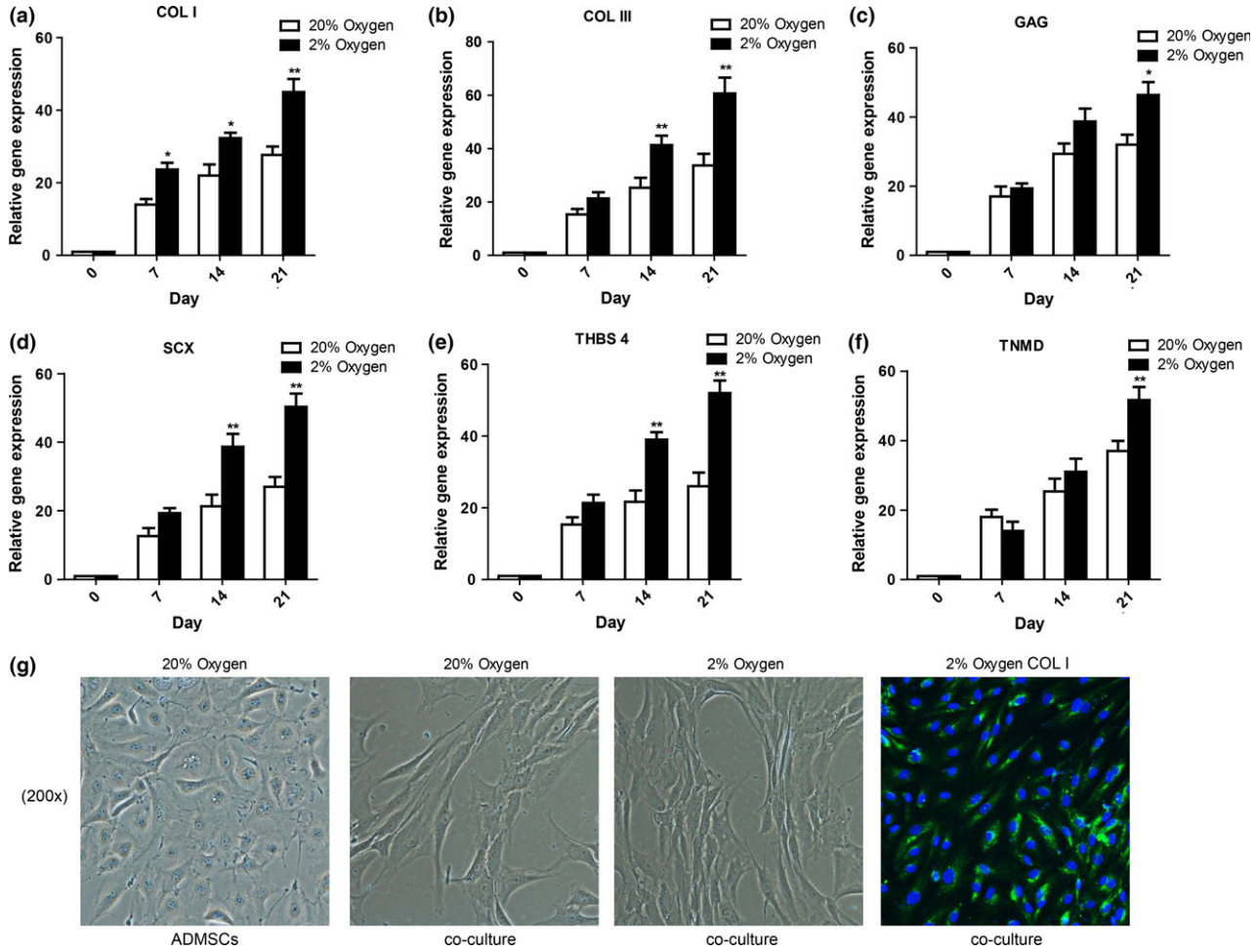

Figure 1. Real-time PCR analysis of gene expression of (a) COL I, (b) COL III, (c) GAG, (d) SCX, (e) THBS 4 and (f) TNMD, with standard error shown. $*P < 0.05$; $**P < 0.01$, compared with control. (g) The morphology of ADMSCs in each groups (original magnification × 200); cells were detected at day 21 post-induction.

significantly higher levels of COL I, COL III, TNMD, THBS 4 and SCX at day 21 post-induction compared to the normoxia control (Fig. 2c,d), indicating that hypoxia promotes the differentiation of ADMSCs towards tenocytes in co-culture. The immunofluorescence staining results of COL I and SCX were consistent with the Western blot results (Fig. 2e).

Hypoxia increases HIF-1α expression in co-culture of ADMSCs with tenocytes

It has been reported that HIF-1α is involved in regulating the crucial cellular processes such as stemness, proliferation and differentiation. In our model, we detected the level of HIF-1α expression by qPCR, Western blot and immunofluorescence respectively. Figure 3a showed that the gene expression of HIF-1α was increased significantly in response to hypoxia as compared with normoxia control. Western blot results showed that the level of HIF-1α was significantly higher in hypoxic culture condition compared with normoxic culture condition (Fig. 3b,c) ($**P < 0.01$). The immunofluorescence staining result of HIF-1α was consistent with the Western blot and qPCR results (Fig. 3d).

HIF-1α inhibitor attenuates the effect of hypoxia on the differentiation of ADMSCs

To further study the role of HIF-1α in the differentiation of ADMSCs to tenocytes in co-culture system, HIF-1α inhibitor (3-[2-(4-adamantan-1-yl-phenoxy)-acetylamino]-4-hydroxybenzoic acid methyl ester; Calbiochem.) was used to block the hypoxia-induced accumulation of cellular HIF-1α protein and then evaluate the differentiation of ADMSCs in hypoxic culture condition. The inhibitor selectively blocks the hypoxia-induced accumu-

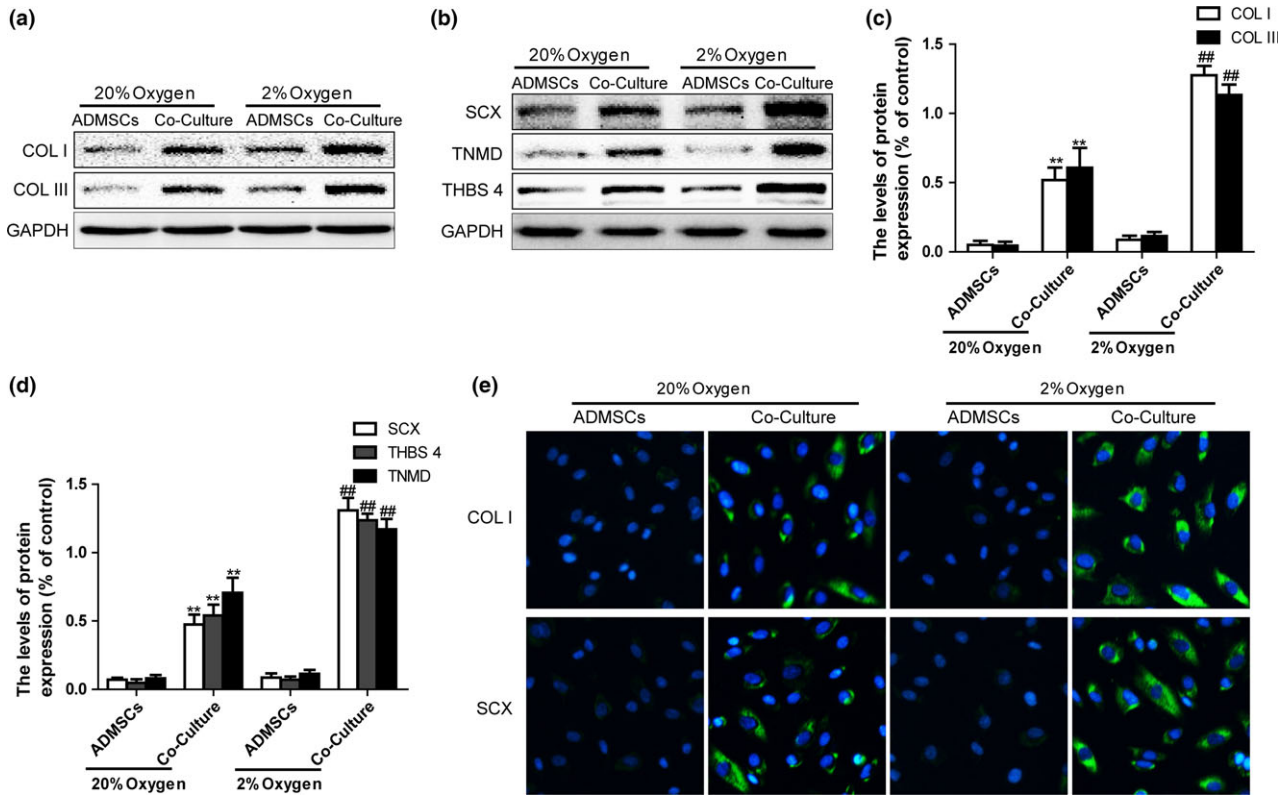

Figure 2. Hypoxia increases the differentiation of ADMSCs to tenocytes in indirect co-culture. (a) Representative Western blots of COL I and COL III in each group. (b) Representative Western blots of SCX, TNMD and THBS 4 in each group. (c, d) Quantification of Western blot data from (a) and (b). All data represent mean values ± SD, $^{**}P < 0.01$, versus the ADMSCs in monoculture group under normoxic culture condition; $^{\#\#}P < 0.01$, versus the ADMSCs in monoculture group under hypoxic culture condition. Representative micrographs showing immunofluorescence (original magnification × 200) of COL I (green) and SCX (green), and DAPI - labelled nuclei (blue) in each group. All cells were detected at day 21 post-induction.

lation of the cellular HIF-1α protein, while exhibiting no apparent effect on the cellular level of HIF-1α mRNA or that of the HIF-1β protein. As shown in Fig. 4a–d, Western blot results showed that the levels of tenocytes-associated proteins markers (COL I, COL III, TNMD, THBS 4 and SCX) were increased in hypoxic culture condition; however, these increases were significantly inhibited in the HIF-1α inhibitor-treated group as compared with the vehicle-treated group in hypoxic culture condition. The results from in vitro experiments showed that HIF-1α inhibitor had no significant effects on protein expression of tenocytes-associated proteins markers compared with the vehicle-treated group in normoxic culture condition (Fig. 4b,d). We next detected the alterations of HIF-1α proteins by Western blot. As shown, there was an increase of HIF-1α expression in hypoxic group, and this increase was inhibited in HIF-1α inhibitor group (Fig. 4e,f). Immunofluorescence also showed that the fluorescence intensity of COL I and HIF-1α immunoreactivity was increased in hypoxic group as compared to normoxic controls, and HIF-1α inhibitor treatment attenuated the increase in its intensity (Fig. 4f). These data suggest that hypoxia increases the differentiation of ADMSCs to tenocytes in indirect co-culture system by inducing the expression of HIF-1α.

Roxadustat (FG-4592) increases the differentiation of ADMSCs in hypoxic conditions

To further study the role of HIF-1α in the tenocytes differentiation of ADMSCs in hypoxic conditions, HIF-1α was up-regulated by using FG-4592 a cell-permeable prolyl-4-hydroxylase inhibitor in vitro. The expression of related tenocytes-associated proteins markers in ADMSCs, treated with or without FG-4592, was determined by Western blot. As shown, the levels of COL I, COL III, TNMD, THBS 4 and SCX were increased in hypoxic groups, and these increase were significantly exacerbated in the FG-4592-treated group as compared with the hypoxic groups (Fig. 5a–d) ($^{*}P < 0.05$, $^{**}P < 0.01$). Next, we detected the level of HIF-1α; qPCR and Western blot results showed that the level of HIF-1α expression was much

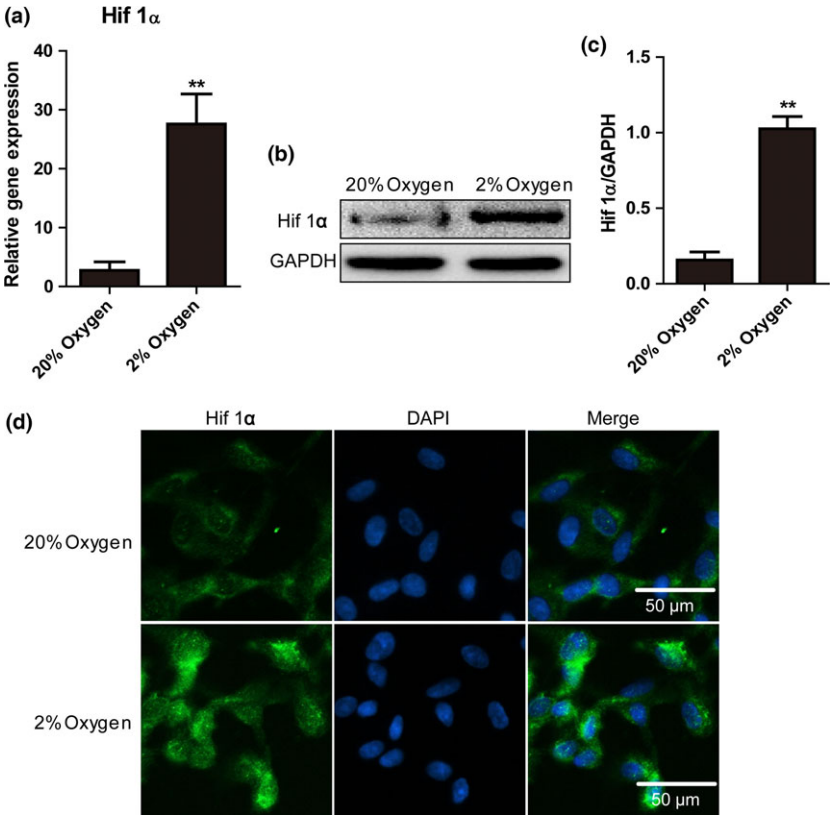

Figure 3. Hypoxia increases HIF-1α expression in co-culture of ADMSCs with tenocytes. (a) The gene expression of HIF-1α in normoxia and hypoxia groups. (b, c) Representative Western blots and quantification data of HIF-1α in normoxia and hypoxia groups, $^{**}P < 0.01$, versus the normoxic culture group. (d) Immunofluorescence staining of HIF-1α (green) in normoxia and hypoxia groups, and DAPI - labelled nuclei (blue). All cells were detected at day 21 post-induction.

higher in the FG-4592-treated group than in the vehicle-treated group (Fig. 5e–g). Immunofluorescence staining results of COL I and HIF-1α are consistent with the Western blot results (Fig. 5h). These results indicate that HIF-1α plays a central role in the differentiation of ADMSCs towards tenocytes in hypoxic conditions.

FG-4592 improves the tenocytes differentiation potential of ADMSCs in normoxic conditions

To verify whether FG-4592 improves the tenocytes differentiation potential of ADMSCs in normoxic conditions, the expression of related tenocytes-associated proteins COL I, COL III, TNMD, THBS 4 and SCX were determined by Western blot. The results of the tenocytes-associated proteins expression (Fig. 6a–d) showed that the levels of COL I, COL III, TNMD, THBS 4 and SCX in ADMSCs were significantly increased after exposure to FG-4592. The level of HIF-1α protein was also enhanced in response to FG-4592 treatment in normoxic conditions (Fig. 6e,f). These findings illustrate that FG-4592 can improve the tenocytes differentiation potential of ADMSCs by activating the expression of HIF-1α in normoxic conditions.

Discussion

ADMSCs have been widely studied in pre-clinical research for the regeneration of musculoskeletal tissue as an alternative to BMSCs, but, until now, only few studies have been performed on tendon tissue (2,14,22–26). Previous studies have demonstrated the role of injected autologous or allogenic ADMSCs in improving tendon mechanical strength, healing, functional recovery, collagen fibre organization and preventing the progression of the lesion and inflammatory infiltrate in vivo (2,13). However, there is little evidence that ADMSCs can differentiate into a tendonogenic lineage in vitro.

Co-culture systems have received much attention because in the co-culture techniques, the stem cells that one wants to differentiate are cultured together with target cells in an effort to promote differentiation due to cellular interactions, such as by trophic effects (27–29). Recent literature reported by Kraus *et al.*, indicated that

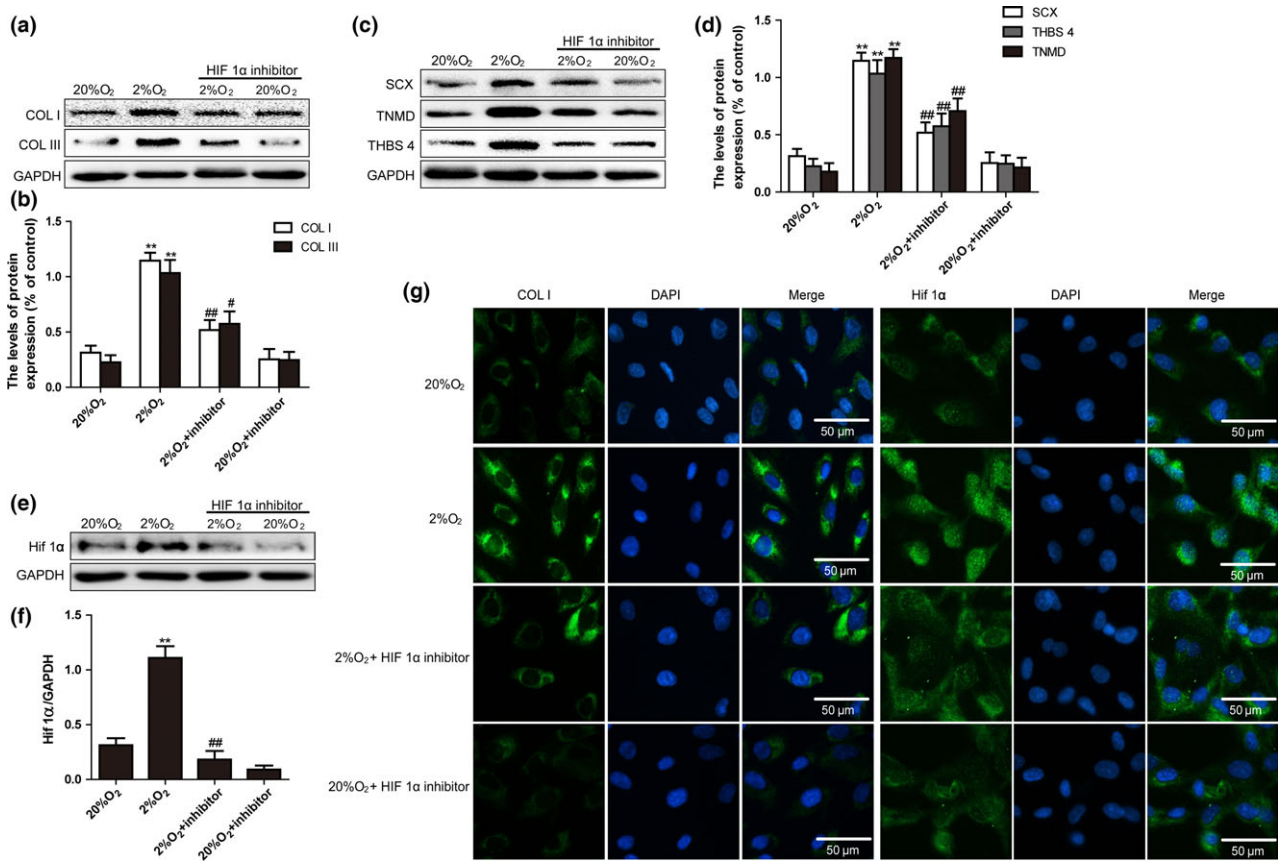

Figure 4. HIF-1α inhibitor attenuates the effect of hypoxia on the differentiation of ADMSCs. (a–d) Representative Western blots and quantification data of COL I, COL III, TNMD, THBS 4 and SCX in each group, $^{**}P < 0.01$, versus the 20% O_2 culture group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus the 2% O_2 culture group. (e, f) Representative Western blots and quantification data of HIF-1α in each group, $^{**}P < 0.01$, versus the 20% O_2 culture group; $^{\#\#}P < 0.01$, versus the 2% O_2 culture group. All data represent mean values ± SD. (g) Representative micrographs showing immunofluorescence of COL I (green) and HIF-1α (green), and DAPI - labelled nuclei (blue) in each group. All cells were detected at day 21 post-induction.

in direct co-culture, tenascin C was up-regulated compared with ADMSCs monoculture, and SCX expression was significantly up-regulated (1.5-fold) in the group with an ADMSCs-to-tenocyte ratio of 1:3. However, for ADMSCs cultured with tenocytes in the indirect co-culture system, a 2.3 ± 0.9-fold up-regulation of tenascin C expression could be detected in ADMSCs, interestingly, there was no significant up-regulation in the expression of SCX, COL I, COL III or elastin compared with ADMSCs in monoculture (29). Contrary to the above, other latter findings indicate that an up-regulation in gene expression of COL I, COL III, tenascin C and SCX in rat BMSCs, in co-culture experiments with rat tenocytes in an indirect co-culture system performed by Luo *et al.* (30). One possibility for this discrepancy is that the human ADMSCs used in Kraus's study are different from rat BMSCs used in Luo's study; however, the precise role of indirect co-culture system in MSCs differentiation remains unclear in various MSCs especially in ADMSCs and warrants further investigation. In the present study, we evaluated the gene expression of tendon-related markers COL1, COL3, TNMD, THBS4, SCX and GAG expression via qPCR at 7, 14 and 21 days, and immunofluorescence staining of COL 1 and SCX and Western blot of COL1, COL3, TNMD, THBS4 and SCX in co-cultural ADMSCs were performed under normoxic conditions at 21 days. After 21 days of transwell co-culture with tendon pieces, the hypoxia-treated cells became more elongated, slender with thinner processes, with a tenocyte-like shape and distribution (Fig. 1g). As shown in Fig. 1, ADMSCs in indirect co-culture with tenocytes showed a trend of time-dependent increase of all tested tenogenic genes expression, and the protein levels of tendon-related markers were significantly increased in indirect co-culture cells compared to ADMSCs in monoculture in normoxia conditions (Fig. 2). The current findings suggest that the use of a co-culture system may be applicable in

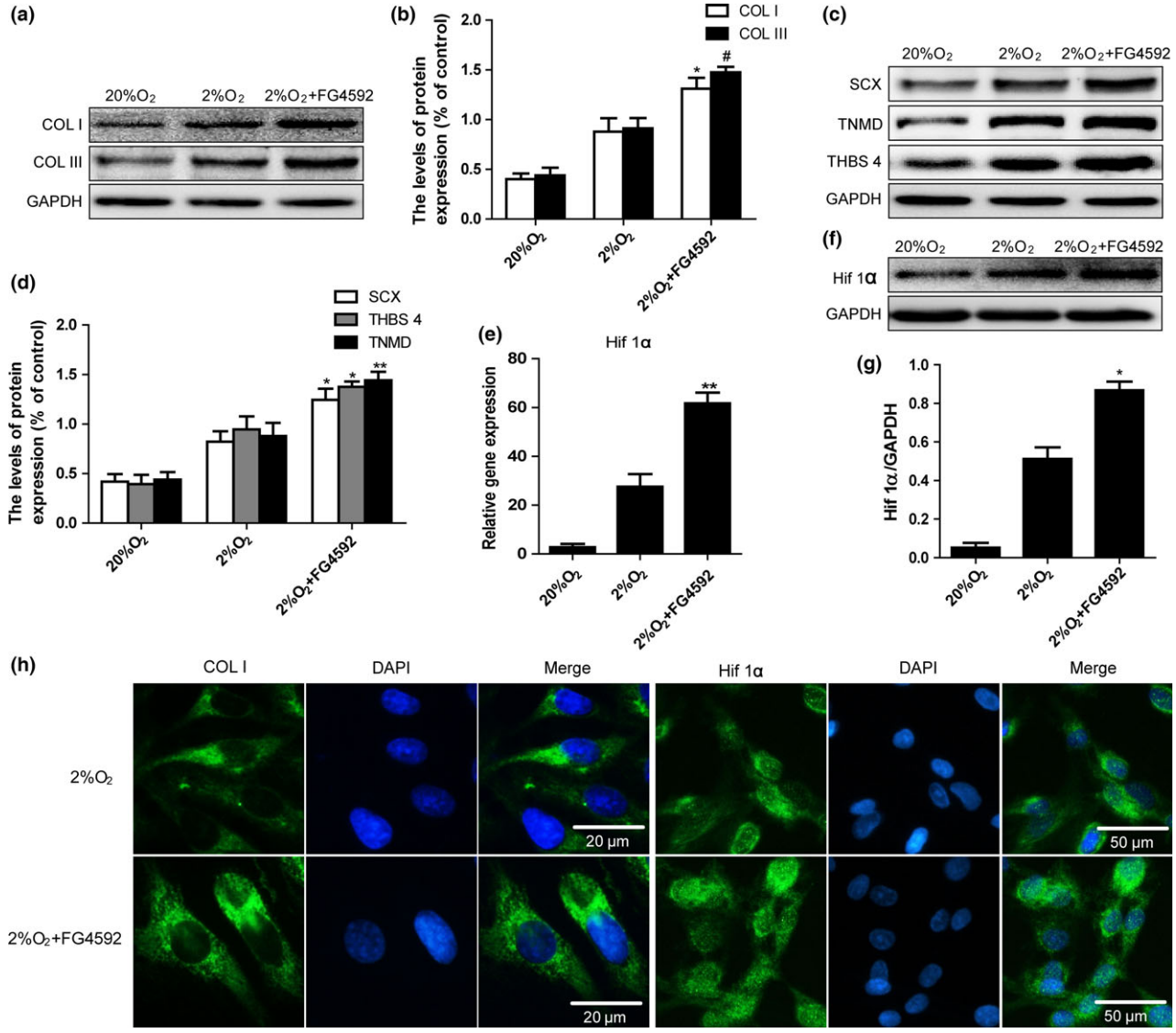

Figure 5. Roxadustat (FG-4592) increases the differentiation of ADMSCs in hypoxic conditions. (a–d) Representative Western blots and quantification data of COL I, COL III, TNMD, THBS 4 and SCX in each group, $*P < 0.05$, $^{\#}P < 0.05$, $**P < 0.01$, versus the 2% O_2 culture group. (e) The gene expression of HIF-1α in each group, $**P < 0.01$, versus the 2% O_2 culture group. (f, g) Representative Western blots and quantification data of HIF-1α in each group, $*P < 0.05$, versus the 2% O_2 culture group. All data represent mean values ± SD. (h) Representative micrographs showing immunofluorescence of COL I (green) and HIF-1α (green), and DAPI - labelled nuclei (blue) in each group. All cells were detected at day 21 post-induction.

future translational use, as both cell types could be harvested from a patient by liposuction and tendon biopsy.

Cultivation in hypoxia has beneficial effects on ADMSCs during in vitro manipulation, conditions that enhance proliferation, migration, and growth factor secretion (15,17,31). In the present study, our data showed that the number of cells cultured under hypoxia was significantly increased as compared to normoxia starting from day 7 of the culture (data not shown). Consistent with other findings, a low oxygen microenvironment provides an optimal condition for the differentiation of ADMSCs (16,19,32,33). In this study, in both normoxia and hypoxia groups, the ADMSCs in indirect co-culture with tenocytes showed a trend of time-dependent increase of all tested tenogenic genes and proteins. As shown in Fig. 1, hypoxia treatment significantly increased the gene expression levels of COL I, COL III, TNMD, THBS 4, SCX and GAG respectively within 7–21 days, 14–21 days, 21 days, 14–21 days, 14–21 days, 21 days after co-culture with tenocytes, compared to normoxia-treated controls ($*P < 0.05$, $**P < 0.01$). From the results of our study, we could find that there

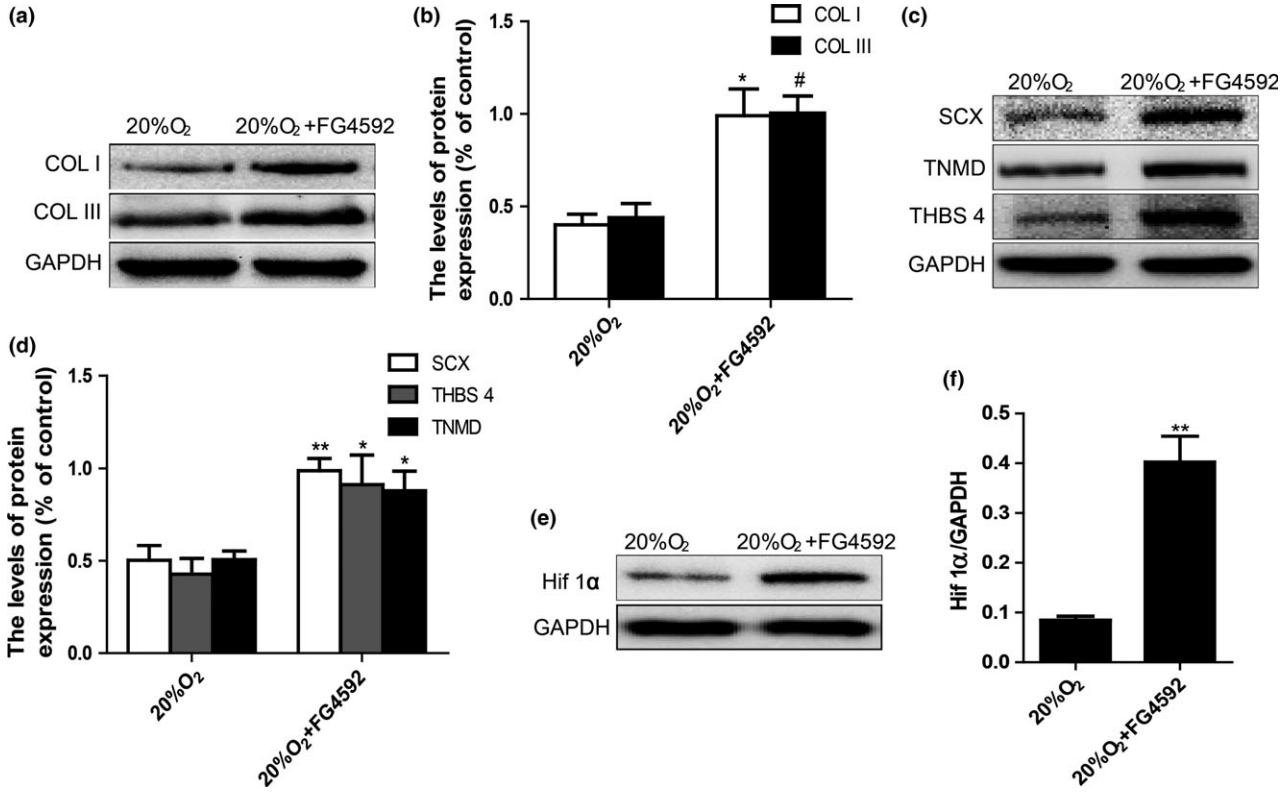

Figure 6. FG-4592 improves the tenocytes differentiation potential of ADMSCs in normoxic conditions. (a–d) Representative Western blots and quantification data of COL I, COL III, TNMD, THBS 4 and SCX in each group, $^{*}P < 0.05$, $^{\#}P < 0.05$, $^{**}P < 0.01$, versus the 20% O_2 culture group. (e, f) Representative Western blots and quantification data of HIF-1α in each group, $^{**}P < 0.01$, versus the 20% O_2 culture group. All data represent mean values ± SD. All cells were detected at day 21 post-induction.

is no significant difference between two oxygen tensions at day 7 point (except COL I), we deduced that hypoxic ADMSCs exhibited an extended lag phase in order to acclimatize to different culture conditions; however, the precise signalling mechanisms remain to be determined.

Hypoxia increases the proliferation and migration of AMDSCs by the generation of reactive oxygen species (ROS) and downstream phosphorylation of the platelet-derived growth factor receptor-beta, ERK1/2 and Akt (34,35). Inhibition of mitochondrial ROS (mtROS) generation reduces the adipocyte differentiation, which suggests that mtROS may be key in hypoxia-induced adipocyte differentiation of ADMSCs (34). Inhibition of the PI3K/Akt/mTOR pathway attenuates the hypoxia-induced adipocyte differentiation. It has been reported that transcriptional factor HIF-1α is involved in regulating the crucial cellular processes such as stemness, proliferation and differentiation (18,36,37). However, conflicting results have been reported regarding the effect of hypoxia on ADMSCs physiological activities, particularly stemness and differentiation. Some of the studies suggested that under hypoxia, HIF-1α enhances the stemness properties of ADMSCs while repressing their differentiation activities (38). In contrary, some demonstrated an increased differentiation potential in a HIF-1α-dependent manner (18,37,39). In the current study, our results indicate that the increased differentiation rate of ADMSCs and high expression of HIF-1α indicated that HIF-1α might play a role in regulating ADMSCs differentiation under hypoxia. Next HIF-1α inhibitor was used to block the hypoxia-induced accumulation of the cellular HIF-1α protein and then evaluate the differentiation of ADMSCs in hypoxic co-culture condition. As shown in Fig. 4a–d, these increases of COL I, COL III, TNMD, THBS 4 and SCX were significantly inhibited in the HIF-1α inhibitor-treated group as compared with the vehicle-treated group in hypoxic co-culture condition. Our findings suggested that HIF-1α plays a critical role in ADMSCs differentiation in the indirect co-culture system under hypoxia.

Prolyl hydroxylase domain (PHD) proteins are known to regulate HIF-1α by hydroxylating two proline residues (Pro-402 and Pro-564) in its α subunit in response to cellular oxygen availability (40). Roxadustat (FG-4592) is a cell-permeable PHD inhibitor, which can up-regulate the protein level of HIF-1α post-transcrip-

tionally under normoxic conditions (21,41). By targeting components of stabilization of HIF-1α signalling responses, to explore clinical treatment strategies or new drugs in tendon injuries demanding multipotent cells for treatment might become possible and valuable in the future. FG-2216, the lead drug candidate, has completed Phase II trials, while the second-generation FG-4592 is currently enrolling for Phase IIa and IIb trials in the United States (21,41). In this study, the results of Western blot and immunofluorescence staining demonstrated that FG-4592 could improve the tenocytes differentiation potential of ADMSCs in vitro by activating the expression of HIF-1α in both hypoxic conditions and normoxic conditions. The present study lays the ground work for future translational confidence of PHD inhibitor such as FG-4592 in tendon injuries. However, much work remains to be done. Whether FG-4592 improves the tenocytes differentiation potential of ADMSCs in vivo? Does FG-4592 in the higher level mammalian like humans and old world monkeys have the therapeutic after tendon injuries? How about the effect of FG-4592 on other stem cells after tendon injuries? These questions are very important for the clinical use of FG-4592 after tendon injuries in the future.

In conclusion, our research demonstrated that the indirect co-culture system is able to induce the differentiation of AMDSCs into tendons-like cells, with a high efficiency both in both hypoxic conditions and normoxic conditions. Furthermore, we confirmed that hypoxia enhances ADMSCs proliferation and differentiation in tenocyte lineages in the presence of co-culture system. In addition, HIF-1α expression was found to be significantly up-regulated during ADMSCs differentiation into tenocytes-like cells, and HIF-1α inhibitor attenuated the effect of hypoxia on the differentiation of ADMSCs. In addition, HIF-1α stabilizator FG-4592 improved the tenocytes differentiation potential of ADMSCs in vitro by activating the expression of HIF-1α in both hypoxic conditions and normoxic conditions. Finally, although further research is still needed, data obtained in this study provide a foundation for the application of ADMSCs in vivo for tendons regeneration.

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Conflict of interest

The authors declare no conflict of interest.

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